TLR-Mediated Loss of CD62L Focuses B Cell Traffic to the Spleen during Salmonella typhimurium Infection

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TLR-mediated loss of CD62L focuses B cell traffic to the spleen during *Salmonella typhimurium* infection[1]

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Abstract

B cells recognise antigens on micro-organisms both with their B cell receptors (BCR) and via Toll-like receptors (TLRs). This innate recognition has the potential to alter the behaviour of whole populations of B cells. We show here that in culture and in mice, MyD88-dependent activation of B cells via TLR2 or TLR9 causes the rapid loss of expression of CD62L, by metalloproteinase-dependent shedding. Adoptive transfer of *in vitro* CpG-activated of B cells showed them to be excluded from lymph nodes and Peyer's patches, but not the spleen. *In vivo*, both injection of CpG and systemic infection with *Salmonella typhimurium* caused the shedding of CD62L and the consequent focusing of B cell migration to the spleen and away from lymph nodes. We propose that wholesale TLR-mediated changes to B cell migration influence the development of immunity to pathogens carrying appropriate ligands.

Keywords

B cell; migration; TLR; Salmonella

Introduction

B cells as antigen presenters and antibody producers are a major component of the adaptive immune response, however, they also have the ability to respond to pathogens in an innate fashion. They do this by recognising structurally conserved pathogen ligands through Toll-like receptors (TLRs) expressed at the cell surface and in the endosomal compartment. Murine B cells are known to express TLRs 1-9 at the mRNA level (1), although there is differential expression in certain subsets. When stimulated through these receptors *in vitro,* B cells are induced to proliferate and differentiate into antibody-secreting plasma cells in a T cell-independent manner (2), while *in vivo* responses to T-dependent antigens also require TLR signalling in B cells for optimal antibody production (3, 4). TLR stimulation of B cells also induces up-regulation of cell surface MHC class II and co-stimulatory molecules (1), enhancing their antigen presenting capacity, and secretion of cytokines such as IL-6, IL-10 and IFNγ (1), allowing the regulation of helper and regulatory T cell responses (5). Therefore, the rapid innate response of B cells to pathogens via TLR stimulation has a direct impact, not only on the developing adaptive B cell response, but also on the magnitude and phenotype of the T helper cell response.

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In order to study TLR-dependent B cell responses *in vivo*, we made use of the murine infection model of typhoid fever, *Salmonella enterica* serovar Typhimurium. Systemic infection by this intracellular Gram-negative bacterium, which resides predominantly in macrophages, results in the development of strong Th1 and antibody responses (6, 7). Early in infection, innate macrophage responses are required to control bacterial growth (8), while specific Th1-associated cytokines (IFNγ, TNFα) are also crucial (9-11). The necessity for TLR signalling for clearance of *Salmonella* infection is well characterised. C3H/HeJ mice, that lack TLR4 expression and so cannot respond to lipopolysaccharide (LPS), are highly susceptible to *Salmonella* infection (12). TLR4 is thought to be important early in infection for cytokine production and killing of bacteria, whereas TLR2 (which recognises bacterial glycolipids and lipopeptides) plays a role later (13). The lack of the MyD88 adaptor protein during the primary immune response to an attenuated strain of *S. typhimurium* results in increased bacterial load, but these mice are able to clear the infection, albeit with delayed kinetics ((14); Barr, T. *et al.,* in press). These mice appear to have impaired IL-12 production and reduced Th1 responses (15), and may in fact mount a skewed Th2 response (14).

The specific role of B cells in the *S. typhimurium* infection model has also been studied (16-18). B cells appear dispensable for the primary immune response to attenuated strains of *Salmonella*, with bacterial load at the peak of infection and the primary T cell response being equivalent in B cell deficient and wild type mice (16). However, protective immunity is absolutely dependent on the presence of B cells, with B cell-deficient mice showing hugely impaired IL-2 and IFNγ production by T cells after bacterial clearance (17), suggesting an absence of T cell memory (17, 18) and in addition, show increased mortality during challenge (17). Transfer of immune serum did not restore protection in B cell-deficient animals, suggesting one important role of B cells in this model is the presentation of antigen to T cells for the generation of memory (17). Indeed, to transfer protection to naïve mice, both immune serum and T cells are required (19).

In our recent work we have addressed the specific role of B cell TLR stimulation in *Salmonella* infection. Using mixed bone marrow chimeras in which the B cell compartment alone is deficient in MyD88 ($MyD88^{B-/-}$) reveals that primary T cell IFNγ production during *S. typhimurium* infection is reduced, suggesting B cells play an important role as antigen presenting cells (APCs) in driving the early Th1 response ((4); TB, SB & DG unpublished). Looking at antibody production, IgG2a/c is reduced in $MyD88^{B-/-}$ mice during *S. typhimurium* infection (4) and this correlates with the impairment of T cell production of IFNγ. Recent work, showing TLR4-mediated changes to splenic structure (20), has highlighted the potential of TLR signalling to affect cell migration. This study started with the observation that certain TLR ligands alter the expression of CD62L on B cells and we characterize here how this affects their migration during infection.

CD62L (L-selectin) is an adhesion molecule belonging to the C-type lectin family that binds carbohydrate ligands, such as those induced on inflamed endothelium and those constitutively expressed at high endothelial venules (HEVs) (21). More specifically, CD62L ligands include sulphated carbohydrates of GlyCAM-1 and CD34 at lymph node HEVs (22), and MAdCAM-1 at Peyer's patch HEVs (23). Binding of CD62L initiates tethering and rolling of cells, and allows the subsequent transmigration from the bloodstream into tissues (24, 25). Blocking antibodies against CD62L have been shown to inhibit lymphocyte binding to HEVs both *in vitro* and *in vivo* (26), while CD62L knockout mice display a 70-90% reduction in lymph node cellularity (27). Naïve lymphocytes, including B cells, are $CD62L^{+}$ and express varying levels of this molecule depending on the organ from which they are isolated. Upon stimulation, by cognate antigen through the B cell receptor (BCR) or by phorbol esters such as PMA, CD62L is shed from the cell surface. The enzyme

responsible for shedding of surface CD62L is a zinc-containing membrane-associated metalloprotease, ADAM17 (also known as TACE), which also cleaves TNFα (28). This cleavage of CD62L by lymphocytes occurs rapidly, with 90% of lymph node cells shedding CD62L in response to PMA within 1 hour (29).

CD62L expression is important in the development of immune responses, as CD62L-deficient mice have reduced leukocyte migration to inflamed sites and impaired delayed-type hypersensitivity responses (30), as well as impaired primary T cell proliferation and cytokine production (31). Furthermore, the metalloprotease-mediated shedding of CD62L is crucial as mice expressing a mutant form that cannot be cleaved from the cell surface exhibited impaired responses to viral infection and delayed viral clearance (32, 33). All of the work to date on CD62L has focussed on T cells and little is known about the consequence of impaired modulation of B cell CD62L.

In this study we found that stimulation of B cells with ligands for TLR2 or TLR9 caused the rapid loss of expression of CD62L and that these cells localised to the spleen, but not the lymph nodes when adoptively transferred iv. We show that similar changes in CD62L expression occur in response to heat-killed *Salmonella* and antigen extracts. Furthermore, during *S. typhimurium* infection, B cells shed CD62L and show altered localisation, increasing numbers in the spleen, with decreasing proportions in lymph nodes and Peyer's patches. This focussing of B cell migration on the spleen may be important for TLR-dependent B cell mediated support for *Salmonella*-specific T cell responses.

Materials and Methods

Mice

C57Bl/6, MyD88$^{-/-}$ (34), TRIF$^{-/-}$ (35), TLR2$^{-/-}$ (36), TLR9$^{-/-}$ (37), and Ly5.1^{+} mice were bred and maintained in specific pathogen-free conditions at the School of Biological Sciences Animal Facility, University of Edinburgh. In all experiments, mice aged 6-10 weeks were used and all genetically modified mice were backcrossed 6-10 generations to C57Bl/6. Experiments were covered by a Project Licence granted by the UK Home Office under the Animals (Scientific Procedures) Act of 1986. Locally, the University of Edinburgh Ethical Review Committee approved this licence.

Cell isolation and B cell purification

Single cell suspensions were prepared from spleen, lymph nodes, and Peyer's patches by manual disruption in Iscove's modified Dulbecco's media (IMDM) plus 5% FCS and penicillin/streptomycin. Following red blood cell lysis, cells were labelled with anti-CD19 microbeads, and sorted over two consecutive LS columns according to the manufacturer's instructions (Miltenyi Biotech, Bisley, UK). Purity was in excess of 98% (data not shown).

In vitro TLR stimulation cultures

Splenocytes or purified B cells were cultured at 4×10^6 cells/mL in complete IMDM in 24 well plates. Endotoxin-free TLR ligands (InVivogen, Autogen Bioclear UK Ltd.) were utilised at the following concentrations: the TLR2 ligands zymosan, peptidoglycan and PAM_3 CSK4 were used at 10, 10 and 0.2 μg/mL, respectively; the TLR3 ligand polyI:C was utilised at 25 μg/mL; LPS (TLR4) from *E. coli* was used at 1 μg/mL; flagellin (TLR5) from *S. typhimurium* was used at 0.1 μg/mL; the TLR7 ligand loxoribine was used at 100 μM; the TLR9 ligand unmethylated CpG DNA (ODN 1826 5′-TCC ATG ACG TTC CTG ACG TT-3′) was used at 5 μg/mL. PMA and ionomycin were used at 10 ng/mL and 1 μg/mL, respectively. Where appropriate, the metalloprotease inhibitor Ro 31-9790 (Roche Research Products, Welwyn Garden City, UK) was used at 50 μg/mL. For the recovery of CD62L

expression, cells were harvested, washed, and plated out again at 4×10^6 cells/mL in fresh media.

In vivo CpG DNA immunisation

C57Bl/6 mice were immunised with 20μg CpG DNA intravenously, or mock immunised in PBS. Mice were bled pre-immunisation and at various times post-immunisation. Lymphocytes were isolated by separation on lympholyte (Cedarlane Labs Ltd, Hornby, Canada) and washed extensively before staining.

Salmonella enterica serovar Typhimurium infection

The aroA attenuated strain of *S. typhimurium* (SL3261) was used for all infections (38). Bacteria were grown as stationary-phase overnight (16 hour) cultures in Luria-Bertani (LB) broth (Difco Laboratories, UK). Animals were injected intravenously with 10^6 colony-forming units, diluted in PBS. Infectious dose was determined by plating bacteria onto LB plates and culturing overnight at 37°C.

Preparation of *S. typhimurium* antigens

Bacterial antigens from *Salmonella enterica* serovar Typhimurium were prepared as previously described (39). Briefly, overnight stationary phase cultures of the SL3261 strain were heat-inactivated at 85°C for 10 minutes. Around 2.5×10^6 CFU bacteria that had undergone heat-inactivation were used for *in vitro* cultures. For B cell culture with live bacteria, around 2.5×10^6 CFU bacteria were used. To prepare the crude sonicate, overnight cultured bacteria (SL3261 strain) were sonicated, and debris removed by centrifugation. To make the C5 antigen, overnight culture of the C5 virulent strain of *S. typhimurium* was sonicated, alkali-treated (NaOH), and neutralised in HCl. Both the crude sonicate and C5 anitgen were used at 20 μg/mL.

In vivo proliferation assay

Mice were injected with 2 mg bromodeoxyuridine (BrdU) intraperitoneally on days 2, 4 and 8 of *Salmonella* infection. The following day (days 3, 5, and 9), spleens were removed and single cell suspensions prepared. Proliferation, as determined by BrdU incorporation, was measured by staining for BrdU using a FITC-BrdU Flow Kit (BD Bioscience) as described in the manufacturer's instructions. Briefly, cells were surface stained with anti-CD19 PE and anti-CD4 APC, washed, then resuspended in fix/perm buffer overnight. The following day, cells were washed and DNase treated for 1 hour at 37°C, then stained with anti-BrdU FITC.

Cell transfers

For cultured cell transfer purified B cells from $Ly5.1^+$ donor mice were cultured with CpG DNA as described above for 4 hours or left unstimulated. After harvesting, cells were washed extensively in PBS, and 5×10^6 cells injected intravenously into recipient C57Bl/6 mice. The $CD62L^{lo}$ phenotype of CpG-stimulated cells was confirmed by flow cytometry. For $CD62L^{hi}$ blood cell transfer: blood was collected into heparin from donor mice by cardiac puncture. Lymphocytes were extracted and washed as described above. 5×10^6 unsorted lymphocytes were injected intravenously into recipient uninfected or day 6 *Salmonella*-infected mice. The $CD62L^{hi}$ phenotype of donor cells was confirmed by flow cytometry.

Flow cytometry

Before staining, cells were washed in FACS buffer (PBS with 0.05% Sodium azide and 3% FCS). The following antibodies were used (all BD Bioscience, SanDiego, CA, unless otherwise stated): anti-CD19-PE, anti-CD4-APC, anti-CD62L-FITC (Abcam, Cambridge,

UK), IgG2a-FITC isotype control (Abcam, Cambridge, UK), anti-Ly5.1-biotin, Streptavidin-PerCP. Cells were stained for 20 minutes on ice and washed 3 times in FACS buffer. Samples were analysed on a FACSCalibur flow cytometer (BD Bioscience, SanDiego, CA) using CellQuest software, and data analysed using FlowJo software (Tree Star Inc, San Carlos, CA).

Statistics

The Student's paired *t*-test was used to calculate significance values where appropriate.

Results

Loss of CD62L expression on B cells in response to some TLR ligands

B cells are known to lose expression of CD62L following the ligation of their BCR (40). We wished to know whether activation via innate receptors, such as TLRs, altered expression of this molecule. To examine the effect of TLR stimulation of B cells on their expression of CD62L, purified B cells were cultured with a variety of TLR ligands *in vitro.* We found that B cells lose expression of CD62L in response to specific TLR ligands, namely PAM_3CSK4 (a TLR2 ligand), CpG DNA (TLR9 ligand), and partially shed in response to polyI:C (a TLR3 ligand). In relation to the latter (TLR3), increasing the dose of polyI:C caused a greater degree of CD62L loss (Supplemental Figure 1), indicating the partial loss is simply a result of efficacy. Stimulation with zymosan, peptidoglycan (both TLR2 ligands), LPS, flagellin or loxoribine (ligands for TLRs 4, 5, and 7, respectively) did not induce shedding (Figure 1A). The highest levels of CD62L expression are found on follicular B cells (Supplemental Figure 1) and peripheral blood B cells (similar profile to follicular B cells; not shown), however, even the low levels seen on marginal zone B cells are significantly modulated by CpG stimulation (Supplemental Figure 1).

Using cells from $MyD88^{-/-}$, $TRIF^{-/-}$, $TLR2^{-/-}$, and $TLR9^{-/-}$ mice, it was confirmed that loss of CD62L on B cells in response to PAM_3 CSK4 is MyD88- and TLR2- dependent, its loss in response to polyI:C is TRIF-dependent, and in response to CpG DNA is MyD88- and TLR9-dependent. All knockout B cells were able to shed when stimulated with PMA/ionomycin (Figure 1B) or by cross-linking their BCR (not shown).

Loss of CD62L expression by B cells stimulated with CpG DNA is rapid and due to shedding from the surface

An analysis of CD62L expression over time after CpG stimulation revealed that B cells lost surface expression of CD62L within 2 hours of culture (Figure 2A). This expression was not restored within the 12 hours analysed. To establish whether B cells lose CD62L expression *in vivo* in response to CpG DNA, mice were injected with 20 μg CpG DNA. As can be seen in Figure 2B, the percentage of B cells in the blood that express high levels of CD62L was significantly reduced at 2, 4, and 8 hours after injection. Expression levels returned to normal by 24 hours post-injection.

It seems likely that the mechanism by which B cells rapidly down-regulate CD62L is by shedding from the surface, as characterised previously with respect to activation by PMA (32). To demonstrate that this was the case, we used the metalloprotease inhibitor Ro 31-9790, which has previously been shown to inhibit CD62L shedding (41). Ro 31-9790 also inhibits shedding of TNFα (42) and is, therefore, thought to target ADAM17 (TACE). When this inhibitor was included in the B cell culture with CpG DNA, the loss of CD62L from the cell surface at the 4-hour time-point was completely abolished (Figure 2C). This confirms that the loss of surface CD62L by B cells in response to CpG DNA was due to shedding by the surface metalloprotease ADAM17 (TACE), and not any other mechanism.

CpG-stimulated B cells are excluded from lymph nodes and Peyer's patches

It is well known that CD62L is essential for leukocyte entry into the lymph nodes across the HEV (26). We predicted, therefore, that CpG-stimulated B cells that have a $CD62L^{lo}$ phenotype would show altered migration patterns *in vivo*. To investigate this, we transferred CpG-stimulated or unstimulated B cells, after 4 hours of culture, into congenic Ly5-distinct hosts, and looked the following day at their localisation. The CpG-stimulated $CD62L^{lo}$ donor cells were excluded from the lymph nodes and from Peyers' patches (Figure 3), but trafficked normally to the spleen. These data indicate that TLR stimulation of B cells can impact on their migration patterns *in vivo* via changes in surface expression levels of CD62L.

B cells recover surface expression of CD62L around 2-3 days after stimulation, and subsequently gain entry into lymph nodes and Peyer's patches

Having established the kinetics of CD62L shedding from the surface of B cells in response to TLR stimulation, we next analysed the recovery of expression both *in vitro* and *in vivo*. Following culture with CpG DNA, B cells were washed extensively and either plated out again with fresh media *in vitro*, or adoptively transferred into Ly5-distinct recipient mice. Figure 4A shows that B cells recover CD62L expression after 3 days in culture and *in vivo* the re-expression happens after 2 days (Figure 4B). The adoptive transfer showed that donor CpG-activated B cells were present in the lymph nodes and Peyer's patches by day 5, in numbers equivalent to unstimulated B cells. The lag of 3 days between CD62L re-expression in the spleen on day 2, and B cell migration to lymph nodes and Peyer's patches on day 5 is likely due to the normal homing patterns of B cells, in that they pause in secondary lymphoid tissues before re-entering the circulation.

B cells shed CD62L when stimulated with antigens from *Salmonella*

To confirm that B cells shed CD62L when encountering TLR ligands in the form of pathogenic bacteria, we stimulated B cells with antigens from the Gram-negative bacteria *Salmonella enterica* serovar Typhimurium (hereafter referred to as *S. typhimurium*). The data presented in Figure 5A reveal that B cells shed CD62L when stimulated with heat-killed bacteria, a crude sonicate of bacteria or a semi-purified C5 antigen. However, no shedding occurred in response to live bacteria *in vitro*.

To determine if a specific TLR and adaptor molecule is responsible for shedding induced by these bacterial antigens, knockout cells were used. The data in Figure 5B show that TLR2 and the adaptor molecule MyD88, rather than TLR9 or TRIF, are utilized to induce shedding in response to these bacterial antigen preparations.

Altered localization of B cells during *S. typhimurium* infection

The data shown so far would predict that TLR-induced changes in CD62L expression will result in altered migration of B cells during systemic infection with *S. typhimurium*. The data presented in Figures 6A & B show that *S. typhimurium* infection caused profound alterations in the localization of B cells. In the lymph nodes and Peyer's patches there was a rapid decrease in both the percentages (Figure 6A) and absolute numbers (Figure 6B, lymph nodes only) of B cells during the first week of infection. This was most apparent in the Peyer's patches, where the proportion of B cells drops from around 80% in an uninfected mouse, to 20% by day 8 of infection. In the spleen, the percentage of B cells remains unchanged at day 4, and is reduced at day 8 (Figure 6A), largely due to an influx of other cells (eg. macrophages). However, looking at absolute numbers (figure 6B), there is actually a 2- to 3-fold increase in splenic B cell numbers at day 4 when compared to an uninfected mouse, and numbers remain elevated at day 8. The increase in B cell numbers in the spleen

at this early stage of infection does not seem to be due to B cell proliferation, as BrdU incorporation by B cells was little above background, and minimal in comparison to T cell division (Figure 6C and D).

The changes in B cell populations seen in lymphoid organs (increase in spleen, reduction in lymph nodes and Peyer's patches) during Salmonella infection are likely due to TLR activation as they are not so apparent in MyD88−/− mice (Supplemental Figure 2). These changes in B cell distribution within lymphoid organs are long lasting (Supplemental Figure 3B). This is likely due to the chronic nature of this infection, in which bacteria not cleared until 6-8 weeks post-infection (Supplemental Figure 3A). Together the data in figure 6 support the notion that B cells shed CD62L in response to bacteria, are from excluded from lymph nodes and consequently enter the spleen in greater numbers, as entry to the spleen is independent of CD62L (43).

CD62L^{hi} B cells shed and migrate to the spleen not lymph nodes when transferred into *Salmonella*-infected mice

The data shown so far suggests that B cells shed CD62L during *S. typhimurium* infection and that this is the cause of altered migration patterns. To address this more directly we isolated CD62L^{hi} B cells from the blood of Ly5.2^{+} donor mice, and transferred them into either *S. typhimurium*-infected or uninfected Ly5.1^{+} recipient mice. Ly5.2^{+} donor B cells were identified and CD62L expression analysed after 18 hours. The results displayed in Figure 7A demonstrate that in uninfected mice, donor Ly5.2^{+} B cells migrated to both the lymph nodes and the spleen, whereas in infected mice the Ly5.2^{+} B cells were found in much greater numbers in the spleen and significantly reduced numbers in the lymph nodes. Donor cells identified in the spleens of uninfected mice have maintained their CD62L^{hi} phenotype, whereas in the spleens of infected mice, donor B cells have shed and are predominantly CD62L^{lo} (Figure 7C). This data confirms that B cells shed CD62L early during *S. typhimurium* infection and that this is related to their enhanced entry into the spleen and relative exclusion from the lymph nodes and Peyer's patches.

Discussion

The data presented here indicate that stimulation of B cells through TLRs 2, 3, and 9 induces shedding of CD62L, which impacts on their migration patterns and results in their exclusion from lymph nodes and Peyer's patches. During *Salmonella typhimurium* infection, this process causes B cells to accumulate in the spleen during the first week.

We found that only certain TLR ligands (PAM_3CSK4, Poly I:C and CpG-DNA) induced shedding of CD62L by B cells *in vitro* (figure 1A). Interestingly, PAM_3CSK4, which binds TLR2/1 hetero-dimers, induced shedding, while other TLR2 ligands, zymosan and peptidoglycan, which bind TLR2/6 hetero-dimers, had no such effect. Differences with the zymosan signal could be explained by its dependence on dectin 1 (44) which is not expressed by B cells (45). The reason is more likely to be due to differential expression of TLR1 and TLR6 by B cells. There is evidence to suggest that although B cells express TLR6 at the mRNA levels (1, 2), they display a greater proliferative response to TLR2/1 stimulation than to TLR2/6 (2), which would support the notion of increased expression of TLR1 compared to TLR6. Therefore, although these two forms of TLR2 signal via the same pathway and so produce the same effects (46), differential expression would account for the differences seen between these stimuli.

Shedding of CD62L by B cells in response to TLR stimulation has not previously been reported. However, other groups have indicated that human neutrophils rapidly lose expression of CD62L following stimulation with TLRs *in vitro* (47, 48), and this method has

been utilised to detect defects in the TLR signalling pathway (47). However, these two articles do not agree on which TLRs induce shedding. On the one hand, Von Bernuth *et al.* suggested that shedding of CD62L by human neutrophils was stimulated by ligands for all the TLRs expressed by granulocytes (TLRs 2/1, 2/6, 4, 5, 7, 8) (47). In contrast, Hayashi *et al.* only saw neutrophil shedding of CD62L in response to TLRs 2/1, 2/6, 4, and 7/8 but not TLRs 3, 5 or 9 (48). The differences seen between these results in relation to TLR5-induced shedding may be due to different mechanisms of extracting neutrophils, or the varying concentrations of flagellin used. These results also contrast to our own data on mouse B cell shedding of CD62L (figure 1A), in that we do not see shedding induced by TLRs 4, 5, or 7. Importantly, in addition to the shedding induced by defined TLR ligands, we also see shedding of CD62L by B cells in response to *Salmonella* antigens (figure 5A). This shedding appears to be largely MyD88-dependent, but not mediated entirely by either TLR2 or TLR9 (figure 5B). However, no shedding was observed in response to live bacteria *in vitro* following 4 hours of culture. This is likely due to the short incubation time of this culture, where there will be little bacterial death (B cells alone are unlikely to induce bacterial killing) and, therefore, only small amounts of accessible TLR ligand.

The TLRs that induce shedding of CD62L by B cells in response to bacteria (TLRs 2, 3, and 9) are not the most abundant or obvious TLR ligands to have an effect during *Salmonella* infection. Although the immune roles of LPS (TLR4) and flagellin (TLR5) in the *Salmonella* infection model have been investigated (49, 50), the TLRs highlighted here have received less attention. Interestingly it has been noted that infection with *S. typhimurium* results in an increase in expression of TLRs 1, 2, and 9 in the infected liver (51), but the spleen was not investigated. Furthermore, incubation of hepatocytes with CpG DNA significantly inhibited the intracellular growth of *S. typhimurium in vitro*, suggesting that stimulation via TLR9 can enhance bacterial killing (52). Rumio *et al.* also demonstrated that pre-treatment with CpG-DNA for 72 hours *in vivo* increases survival when mice are then infected with virulent *S. typhimurium* (53), again suggesting a role for this TLR in inducing bacterial killing. The authors propose that this increased survival is due to the responses of Paneth cells, although they did not investigate this directly. This effect may be partly due to the TLR9-induced changes in B cell localisation and activation. How B cells would be activated by bacterial DNA during infection remains to be clarified, as it seems that unless it is released as “soluble” material following bacterial cell death, the bacterial particles would require receptor (BCR)-mediated uptake (54, 55) and so only *Salmonella*-specific B cells might be activated via TLR9.

The TLR-induced shedding of CD62L by B cells had a profound impact on their migration *in vivo*. These TLR-stimulated B cells are completely excluded from the lymph nodes and Peyer’s patches, and traffic only to the spleen (Figure 3). Our data here suggest that, following a single stimulation with TLR ligands, B cell CD62L returns to normal levels in 2-3 days, and these cells are then able to traffic normally to the lymph nodes and Peyer’s patches (Figure 4). Others have shown that, in T cells, activation by anti-CD3 induces a similar short-term reduction in surface CD62L, with normal levels achieved 2-3 days later (56, 57). This is followed 7 days later by full activation of T cells and down-regulation of surface CD62L as a result of transcriptional regulation (57). In the *Salmonella* infection model, we see that the reduction of B cell numbers in the lymph nodes and Peyer’s patches is only apparent for the first 8 days of infection, and thereafter B cells numbers in these organs begin to return to normal. In the spleen, B cell numbers are elevated on days 4 and 8, but not from day 12 onwards. This would suggest that the focussing of B cells to the spleen by TLR-induced changes in CD62L expression, as seen in *Salmonella* infection, is a short-term phenomenon.

The data presented indicate that, during *Salmonella* infection, TLR stimulation of B cells induces a short-term reduction in levels of surface CD62L, which in turn results in increased B cell trafficking to the spleen (Figure 6). This may be a mechanism to non-specifically attract a polyclonal population of B cells to the spleen. We propose that this will have two effects. First, it will enhance the BCR-dependent activation/selection of antigen-specific B cells in the spleen, both by exposing greater numbers of B cells to bacterial antigens but also by TLR-mediated augmentation of expression of MHC class II and co-stimulatory molecules (58-60). Second, the subsequent polyclonal activation by these and other TLR ligands, will drive B cell cytokine production (1). This cytokine release by B cells has a significant effect on the programming of the early, primary CD4 T cells response (TB, SB & DG unpublished). In addition, this non-specific accumulation of B cells in the spleen may enhance the secretion of natural antibody directly to the site where the bacteria have amassed. Therefore, TLR-induced IgM production in the spleen could bind to the bacteria and contribute to the initiation of the primary response as has been reported (61, 62), and ultimately enhance bacterial killing.

Whether the TLR/CD62L-mediated changes to B cell migration enhance the initiation of the adaptive immune response to *Salmonella* or whether TLR-activated B cells may also play roles in the regulation of the inflammatory response (63-65) or changes to the lymphoid tissue structure during infection (20, 66), the global change in migration behaviour upon TLR ligation seems likely to have significant consequences for the immuno-pathology of this infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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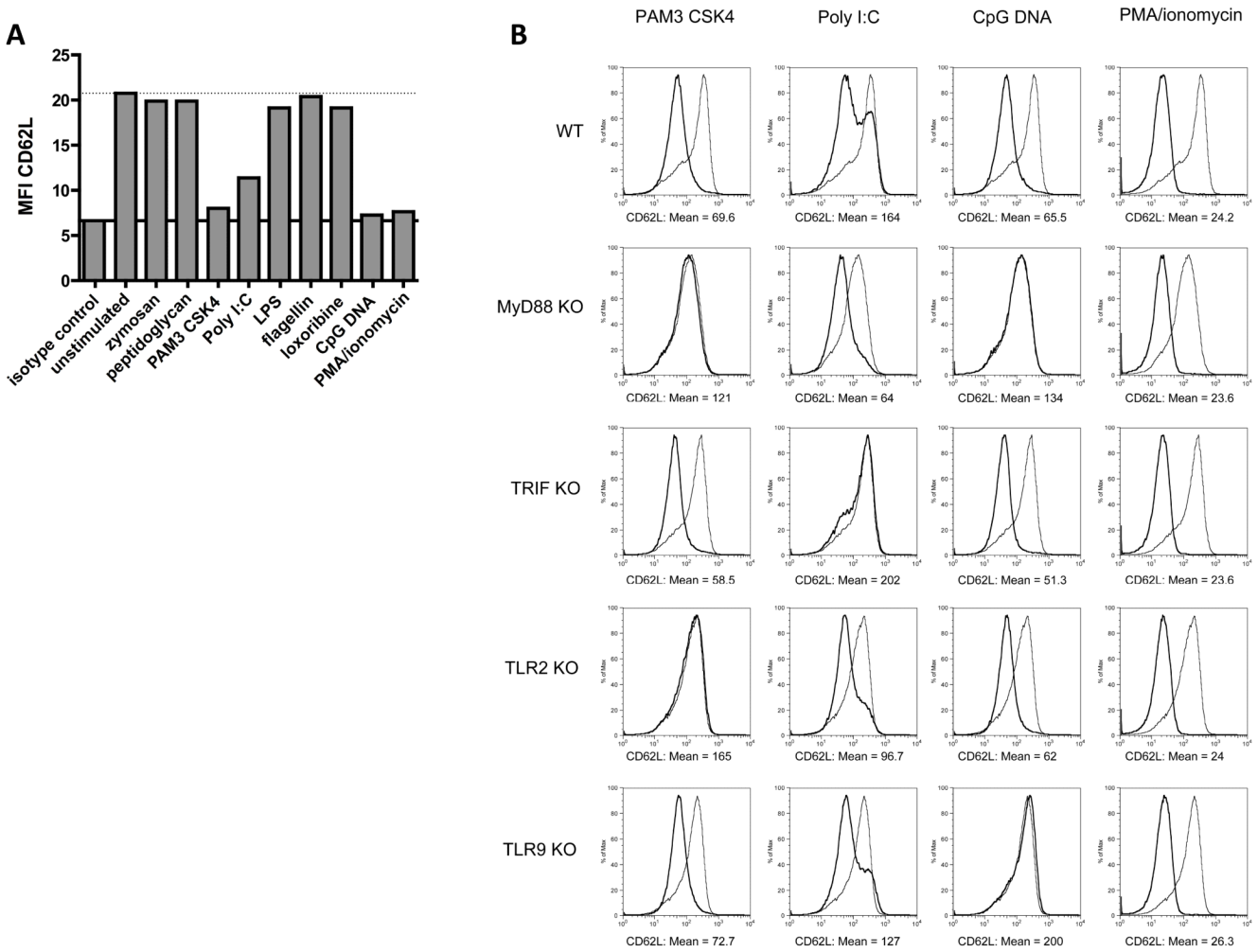

Figure 1. B cells rapidly shed CD62L in response to some TLR ligands

Purified splenic B cells from C57Bl/6 (A, B), MyD88$^{-/-}$, TRIF$^{-/-}$, TLR2$^{-/-}$, and TLR9$^{-/-}$ mice (B) were cultured with a variety of TLR ligands for 4 hours. B cell expression of CD62L was analysed by flow cytometry. PMA/ionomycin is included as a positive control. KO = knockout. In (A) dotted line represents unstimulated, bold line represents isotype control. In (B) dashed line represents unstimulated, bold line shows TLR stimulated. The data presented is from a single experiment using one spleen from each of the knockout mice. Data is representative of 4 independent experiments.

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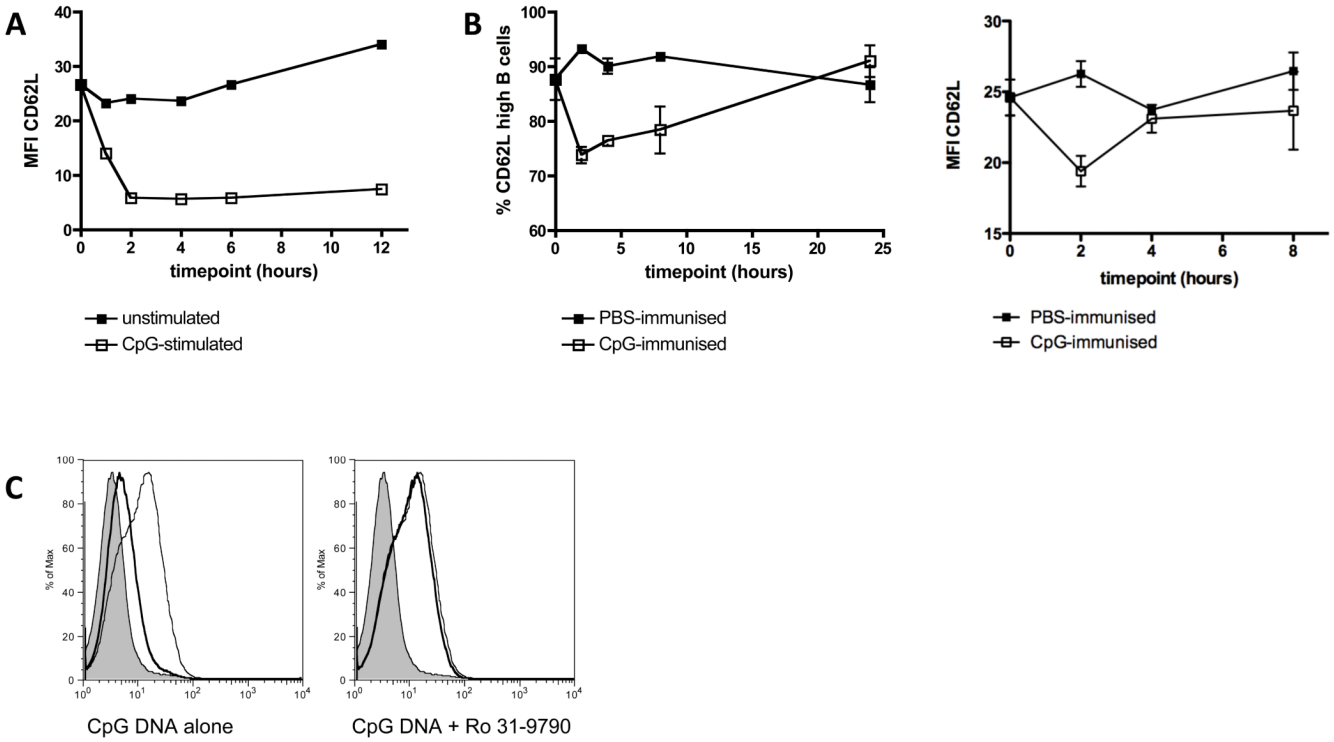

Figure 2. B cell shedding of CD62L in response to CpG DNA is rapid both *in vitro* and *in vivo* and is inhibited by a metalloprotease inhibitor

A) Purified B cells were cultured with 5 μg/mL CpG DNA for up to 12 hours. B) C57Bl/6 mice were injected with 20 μg CpG DNA and bled 2, 4, 8, and 24 hours later. B cell CD62L was identified both by % $CD62L^{hi}$ cells (left) and MFI (right). C) Purified B cells were cultured with CpG DNA for 4 hours with or without the addition of 50 μg/mL metalloprotease inhibitor Ro 31-9790. The isotype control is shaded, unstimulated is in dashed line, and CpG stimulation (+/− Ro 31-9790) is in bold. In all cases, B cells were stained for expression of CD62L and analysed by flow cytometry. Data in (A) and (C) is from an experiment using a single spleen, and is representative of 3 independent experiments. In (B), error bars display SEM, with 5 mice per group, and are representative of 3 experiments.

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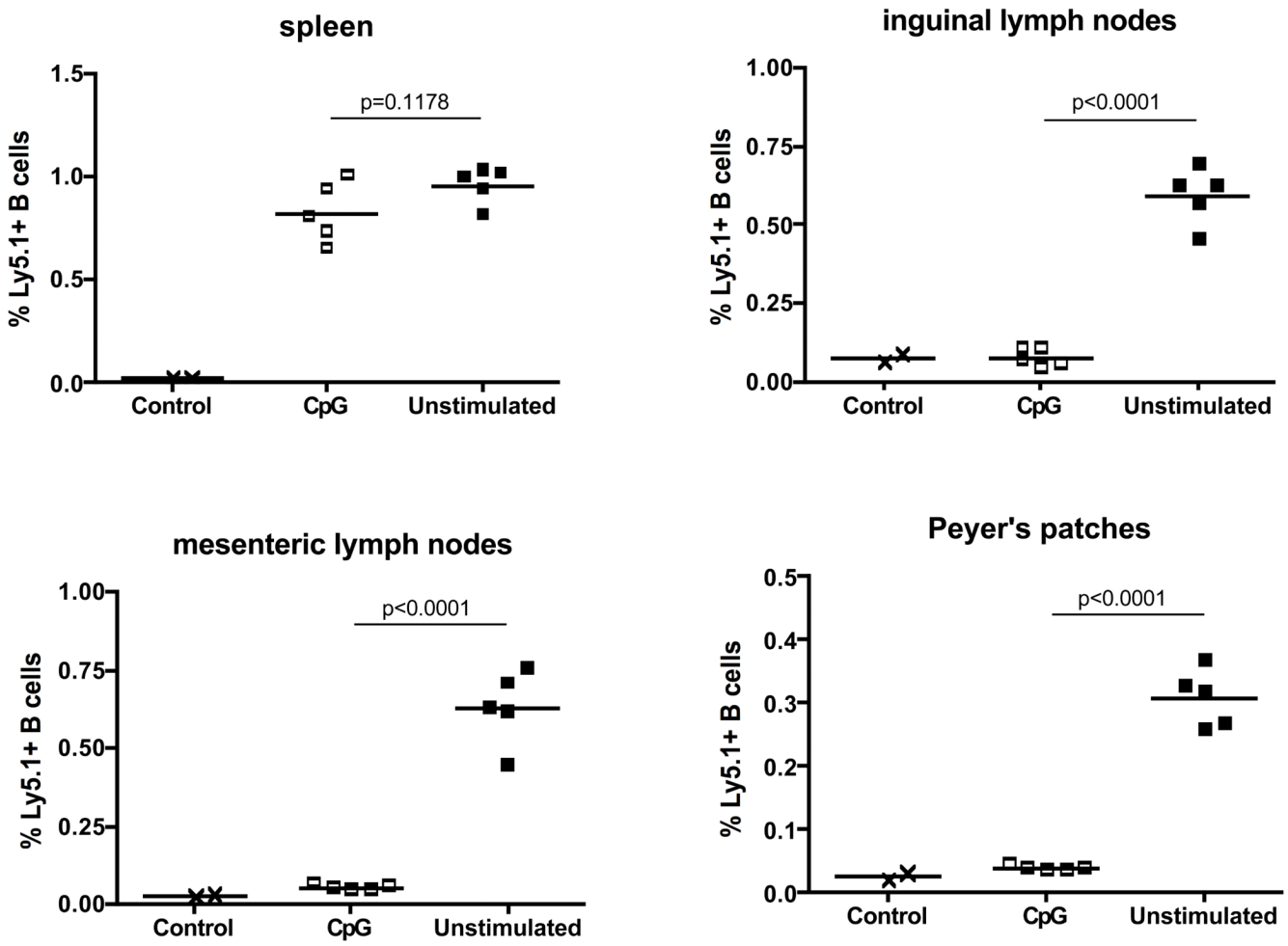

Figure 3. CpG-stimulated B cells are excluded from the lymph nodes and Peyer's patches

Purified Ly5.1$^+$ B cells were cultured with 5μg/mL CpG DNA for 4 hours or left unstimulated. Cells were harvested, washed, and five million cells were then transferred into C57 BL/6 mice. The following day (~18 hours after transfer) organs were taken and donor cells identified. The control mice did not receive cell transfers so indicates background staining. Significance values were calculated using the student's paired *t*-test.

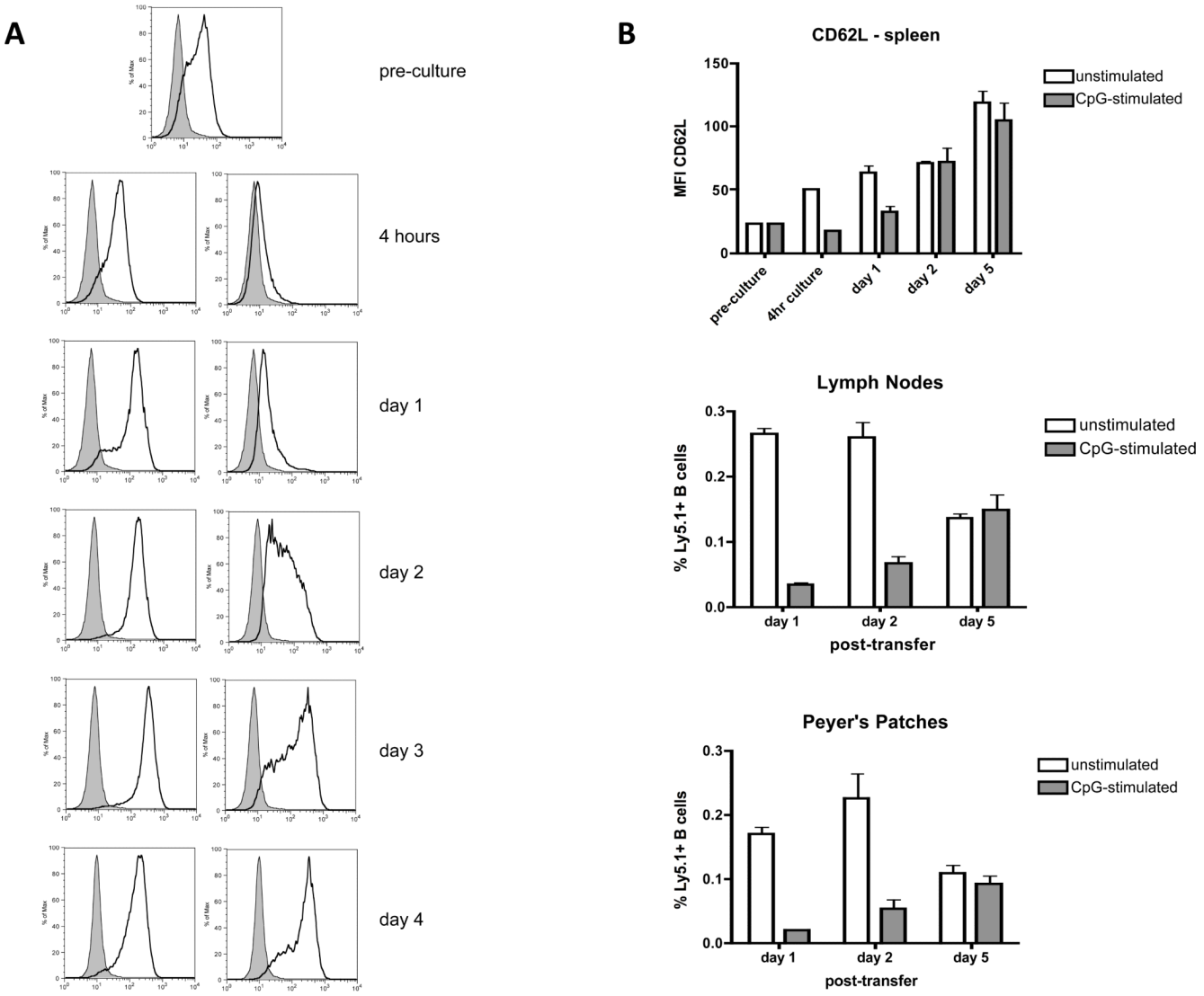

Figure 4. Pattern of CD62L recovery on B cells both *in vitro* and *in vivo*

B cells were stimulated with CpG DNA for 4 hours *in vitro*. A) Cells were harvested, washed and plated out again. Samples were taken up to 4 days later and analysed for B cell expression of CD62L. Left = unstimulated, right = CpG-stimulated. B) After washing, 5 million cells were transferred into Ly5-distinct mice. Over the following days, organs were removed, donor cells identified and CD62L expression levels analysed. Error bars represent SEM, with 4 mice per group. Data is representative of 3 experiments.

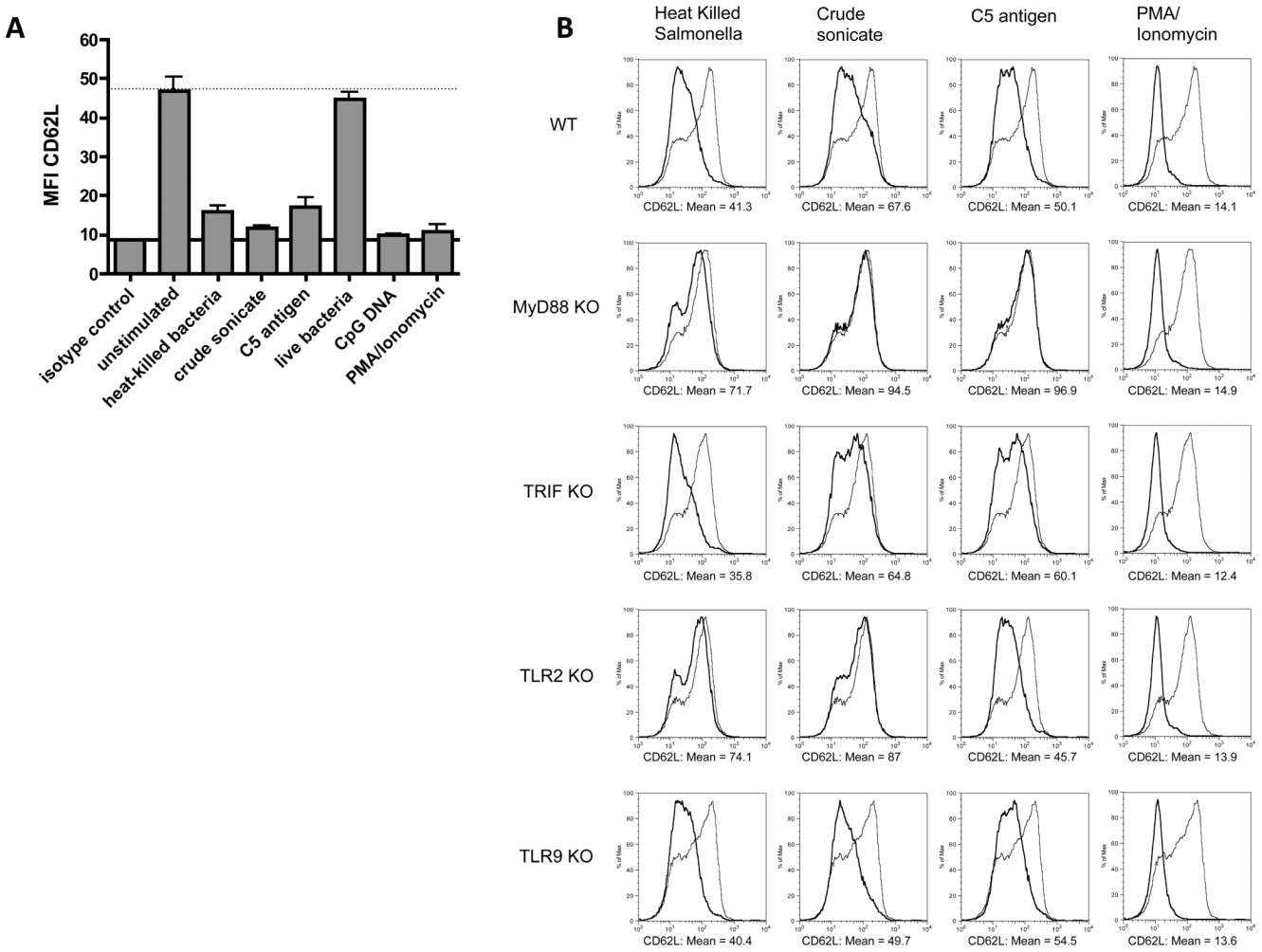

Figure 5. B cells rapidly shed CD62L in response to *S. typhimurium* antigens

Purified B cells from C57Bl/6 (A, B), MyD88$^{-/-}$, TRIF$^{-/-}$, TLR2$^{-/-}$, and TLR9$^{-/-}$ mice (B) were cultured with a variety of antigens from *S. typhimurium* for 4 hours. B cell expression of CD62L was analysed by flow cytometry. In (A) dotted line represents unstimulated, bold line represents isotype control. Error bars indicate SEM. In (B) dashed line represents unstimulated, bold line shows stimulated cells.

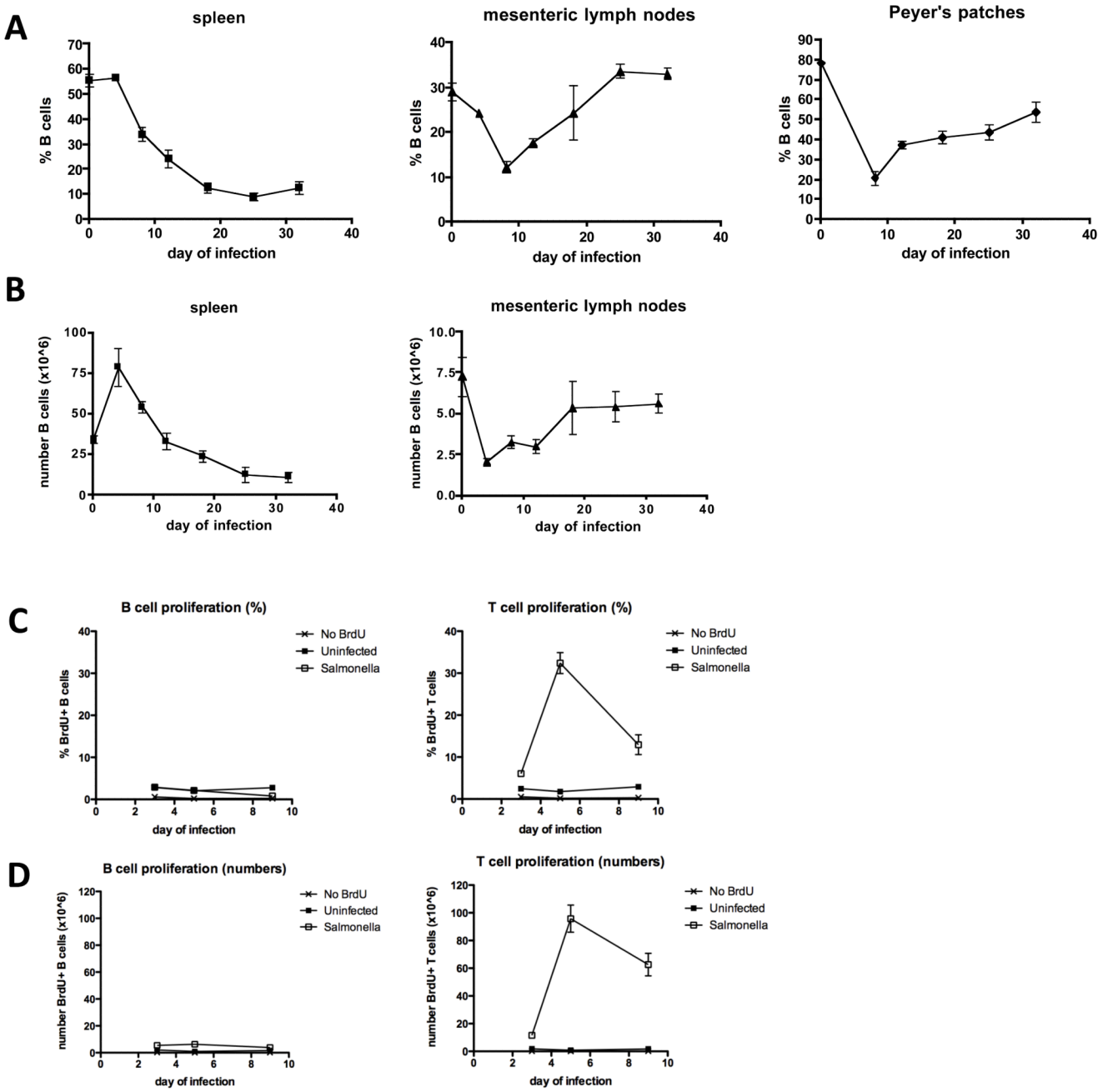

Figure 6. B cell localisation is altered during *S. typhimurium* infection

C57Bl/6 mice were infected with the attenuated SL3261 strain of *S. typhimurium*. Spleens, mesenteric lymph nodes and Peyer's patches were taken at various times during infection and percentages (A) and absolute numbers (B) of B cells identified. Some mice were injected (at either day 2, 4, or 8) with 2 mg BrdU i.p. and splenocytes analysed the following day for percentages (C) and absolute numbers (D) of proliferating cells. Error bars indicate SEM, with 4 mice per group.

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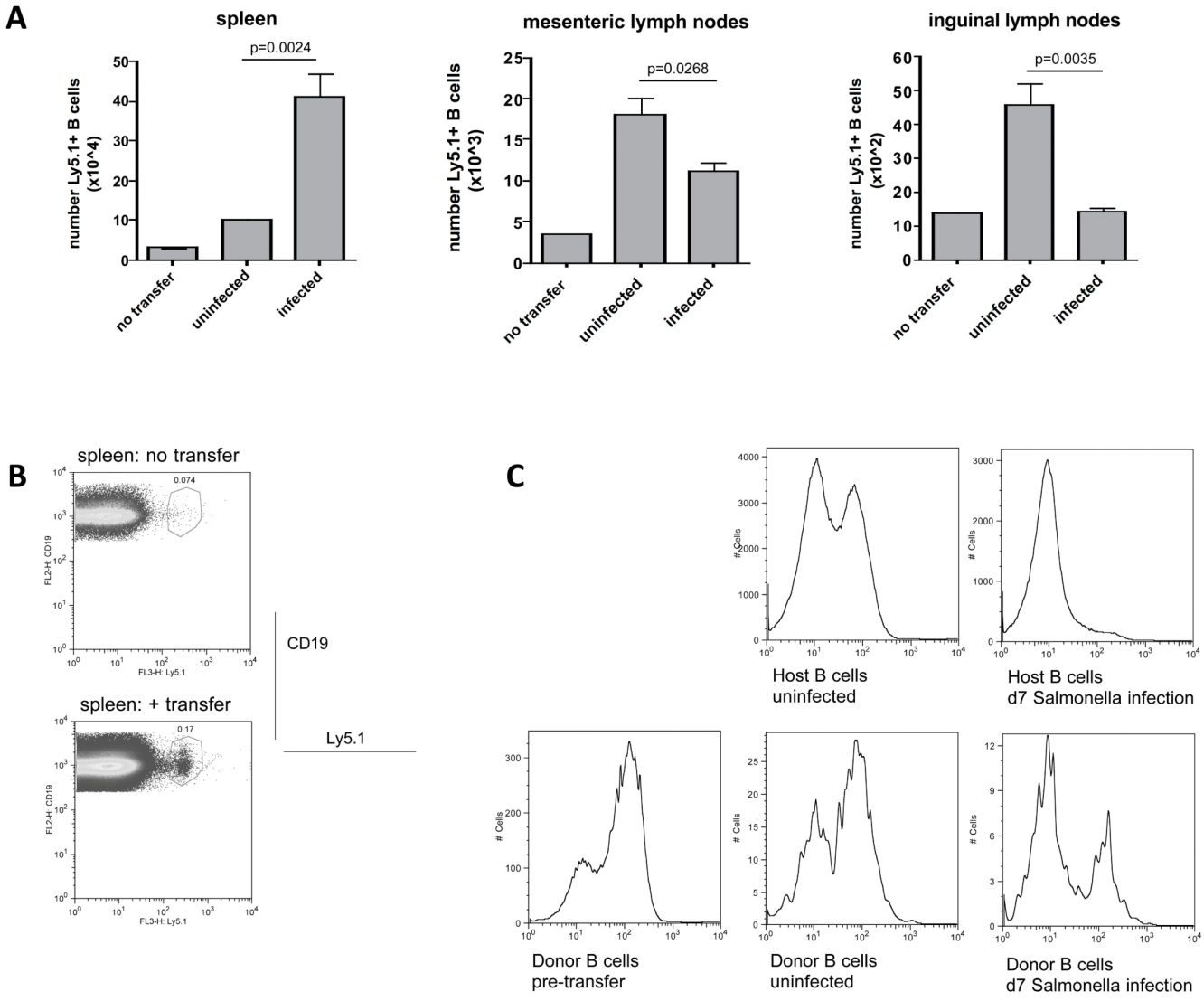

Figure 7. B cells shed CD62L *in vivo* in response to *S. typhimurium*

$CD62L^{hi}$ cells from the blood of $Ly5.1^+$ mice were transferred into either uninfected or *Salmonella*-infected C57Bl/6 mice. The following day, spleens and lymph nodes were removed from recipient mice and donor cells identified (A). Example staining of donor $Ly5.1^+$ cells is presented in (B), showing gating and low-level background staining in mice receiving no donor cells. CD62L expression of splenic B cells was also analysed (C). The student's paired *t*-test was used to calculate significance values. Error bars indicate SEM, with 4 mice per group. Data is representative of 3 independent experiments.